AD_____

Award Number: DAMD17-99-1-9500

TITLE: NF-1 - Regulated Adenylyl Cyclase/c AMP Pathways

PRINCIPAL INVESTIGATOR: Dr. Yi Zhong

CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

REPORT DATE: October 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2001	Annual (1 Sep 00 - 1 Sep 01)

4. TITLE AND SUBTITLE
NF-1 – Regulated Adenylyl Cyclase/c AMP Pathways

5. FUNDING NUMBERS
DAMD17-99-1-9500

6. AUTHOR(S)
Dr. Yi Zhong

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

E-Mail: **zhongyi@cshl.org**

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

This project takes Drosophila NF1 mutants and mouse *Nf1* mutants as models to investigate NF1-dependent regulation of the cAMP pathway. The study is intended to expand the knowledge of the genes that contribute to NF beyond the GAP-related domain in NF1. In the last year, our work has been mainly focused on two aspects. First, the effort has been devoted to determine whether AC activity can be regulated by NF1 in vertebrates. Second, we investigated whether Ras can regulate AC activity in Drosophila. These studies have led to findings that expression of the human NF1 gene was capable of rescuing AC defects in Drosophila and G protein stimulated AC activity is reduced in mouse Nf1 knockout. It was also revealed that Ras, but not Ras-like small G proteins, was able to stimulate AC activity in a NF1 dependent manner. We are continuing to pursue a molecular understanding of this regulation and whether this pathway contributes to pathogenesis of NF1.

14. SUBJECT TERMS
NF1, cAMP, learning, mutant

15. NUMBER OF PAGES
30

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:

The proposed research aims to investigate how the neurofibromatosis 1 (NF1) protein regulates adenylyl cyclase activity in Drosophila and in mice. This pathway has been shown to be critical for mediating a neuropeptide response, cell-size control, and learning and memory in *Drosophila*. Three specific aims have been proposed. (I) Biochemical assay is used for investigation of how G-protein dependent activation of AC is affected by NF1 and whether small G protein Ras is involved in this regulation. (II) We will use molecular and genetic approaches to characterize a newly identified protein, which interacts specifically with both NF1 and Rut-AC. (III) NF1-regulated AC activity will be examined in mice. Since scientific reviewers of this proposal have recommended to remove the second specific aim entirely, our effort will directed only to specific aims I and III. For last yeas, we have mainly examined how G-protein-dependent activation of AC activity is regulated by NF1 in mice and whether Ras can regulate AC activity in a NF1-dependent manner. The results are summarized below.

Body:

1. AC activity regulated by NF1 in mice

Over last year, we have completed experiments to show that AC activity can also be regulated in vertebrates. The manuscript that reports these experiments has been accepted for publication in Nature Neuroscience (in press). Since a large body of data has been presented in the last year report, I will just simply summarize the findings as enlisted below:

(a) Rescue of fly NF1 phenotypes by expression of the human NF1 transgene. We have made transgenic flies that carry humanNF1 (hNF1) transgene under the control of UAS promoter. Expression of the UAS-hNF1 transgene was induced universally in a cross with another transgenic flies expressing Gal4 molecules. It was found that the NF1 mutant defects of small body-size (The et al., 1997) and reduced AC activity in response to GTPγS stimulation (Guo et al., 2000) were largely rescued by expression of hNF1. Since both phenotypes are related to the cAMP pathway, it is suggested that NF1 function in regulation of AC activity is conserved from Drosophila to human.

(b) Alteration of stimulated AC activity in mouse Nf1 knock-out. Assay of AC activity from the membrane fraction extracted from brain tissues at embryonic stage E12.5 indicated that NF1 mutations in mice had a similar effect on stimulated AC as that observed in flies. The basal level of AC activity in homozygous mice knockouts was slightly lower that that in wild type, but showed no difference from heterozygous. However, GTPγS and neuropeptide PACAP38 (pituitary adenylyl cyclase-activating polypeptide)-stimulated AC activity was significantly reduced in the homozygous mutants compared to both wild type and heterozygous embryos, suggesting that G-protein dependent activation of AC was affected in mouse Nf1 mutant too. To determine whether expression of AC was a factor, forskolin stimulated AC activity was assayed. Forskolin is an agent that can, bypassing G protein, stimulate AC activity directly. It was shown that stimulated AC activity in homozygous Nf1 mutant mice was similar to that in heterozygous, but both was slightly lower that that in wild type mice. This data suggests that Acs are present in mutant mice, but failed to be activated normally by G-protein mediated mechanism. Considering homozygous Nf1 mice die

at E13.5 and the observed defect may result from effect of the death, AC activity from cultured neurons was assayed too. A similar defect was observed in cultured Nf1 homozygous mutant neurons in a comparison to heterozygous and wild type neurons.

1. NF1-dependent Ras regulation of AC activity in Drosophila.

(a) Although it has been shown that NF1 is involved in regulation of AC activity, it remains to be determined whether defect in this NF1-regulated AC pathway contributes to pathogenesis or not. We are approaching this problem from two angles. First, we are examining whether and how NF1-regulated AC activity is affected by mutations identified from patients. This study has been supported by a NIH grant. Second, we are looking into whether Ras is able to cross-talk with the AC pathway in a NF1 dependent manner since Ras activity has been considered to play a prominent role in NF1 pathogenesis. We showed that human H-Ras proteins, but not other Ras-like small G proteins, such as Rab3a, were able to stimulated AC activity (Fig. 1). This Ras-dependent stimulation requires NF1 because Ras failed to increase AC activity in NF1 mutants (Fig. 2). We also showed that addition of purified NF1 fragment that contains GRD domain was able to reduce AC activity (Fig. 3). This last result is consistent with the idea that reduction in Ras activity (resulting from addition of NF1-GRD fragments) leads to attenuation of AC activity. Thus, we obtained data to support that Ras is able to regulate AC activity in the NF1-dependent manner.

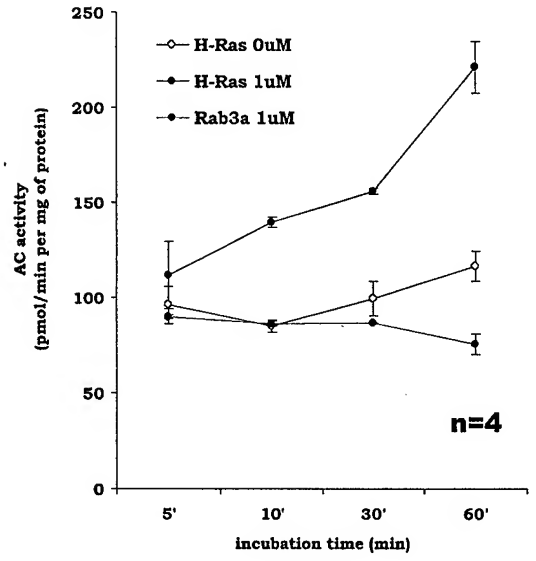


Fig.1 Enhancement of AC activity by H-Ras but not by Rab3a. The membrane fraction of fly brain extracts from wild-type flies was prepared as described (Guo et. al., 2000). Wild type human H-Ras and Rab3a was incubated with the purified membrane extract with time indicated and AC activity was assayed.

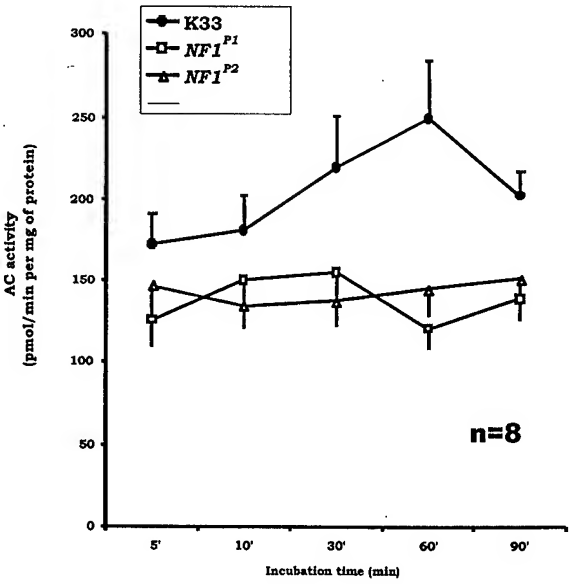


Fig. 2 Lack of Ras-stimulated AC activity in NF1 mutants. 1 μM H-Ras was added to membrane fractions extracted from different genotypes. Time of incubation is as indicated.

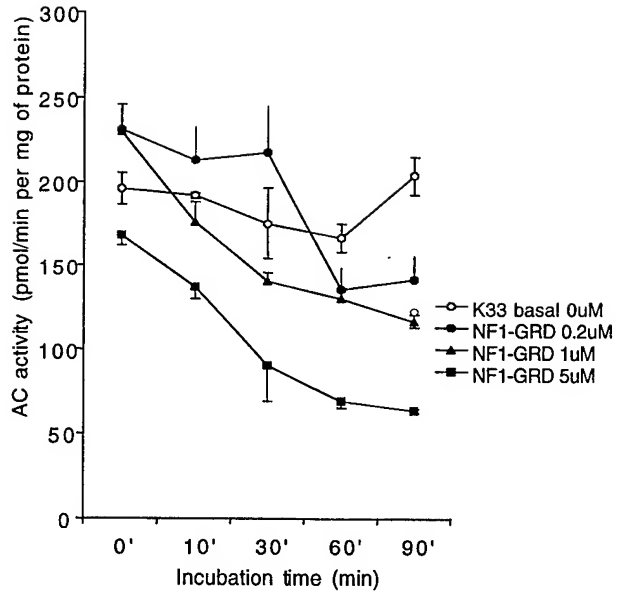


Fig. 3 Inhibition of AC activity by NF1-GRD fragment. Different concentrations of NF1-GRD were added to membrane fraction. Incubation time before AC assay is indicated.

Key Research Accomplishments:

(1) NF1-dependent AC activity is observed in vertebrates.

(2) Ras is able to regulate AC activity in NF1-dependent manner.

Reportable Outcomes:

1. A publication in press in Nature Neuroscience (see Appendices 1)
2. Presentations in the annual meeting for Society for Neuroscience in 2001 held at San Diego (for abstract see Appendices 2) and a presentation in NF meeting held at Aspen in 2001.

Conclusion:

Over last year, we have established a role for NF1 in regulation of AC activity in vertebrates and has obtained strong evidence in supporting a role for Ras in stimulating AC activity in a NF1 dependent manner. We will continue our efforts to determine molecular mechanisms by which NF1 regulates AC activity and whether and how such signal transduction pathway contributes to pathogenesis of NF1.

References:

Guo, H.-F., The, I., Hannan, F., Hariharan, I. K., Bernards, A., and Zhong, Y. (1997) Requirement of *Drosophila* NF1 for activation of adenylyl cyclase by PACAP38-like neuropeptides. *Science* 276: 795-798.

The, I., Hannigan, G. E. Reginald, S., Zhong, Y. Gusella, J. F., Hariharan, I. K. and Bernards, A. (1997) *Drosophila NF1* acts in growth regulation and PKA-mediated signaling. *Science* 276: 791-794.

Guo, H.-F. Tong, T., Hannan, F., Luo, L. and Zhong, Y. (2000) A neurofibromatosis-1-regulated pathway is required for learning in Drosophila. Nature 403: 895-898

Appendices:

1. Tong, J., Hannan, F., Zhu, Y. Bernards, A., & Zhong, Y. Neurofibromin regulates G protein-stimulated adenylyl cyclase activity. Nature Neuroscience (in press).
2. Tong, T. (2001) Neurofibromatosis-1 related aging and sress behavior. Abst. Annul Meeting of Soc. for Neurosc. 27:780.1.

Neurofibromin regulates G protein-stimulated adenylyl cyclase activity

Jiayuan Tong[1,2*], Frances Hannan[1*], Yinghua Zhu[1], Andre Bernards[3], & Yi Zhong[1‡]

1 Cold Spring Harbor Laboratory
P.O.Box 100, Cold Spring Harbor, NY 11724

2 Dept of Neurobiology & Behavior
SUNY at Stony Brook, Stony Brook, NY 11790

3 Massachusetts General Hospital, Cancer Center
Building 149, 13 St. Charlstown, MA 02129

* These two authors contributed equally to this work.

‡ To whom correspondence should be addressed.

Phone (516) 367-6811, FAX (516) 367-8880, Email zhongyi@cshl.org

Neurofibromatosis type 1 (*NF1*) is a dominant genetic disorder that is characterized by formation of multiple benign and malignant tumors in the peripheral and central nervous systems as well as other symptoms, including learning defects in about 45% of children with *NF1* (for review see ref. 1,2). Genetic analysis has indeed confirmed participation of the *NF1*-encoded protein, neurofibromin, in tumorigenesis among mouse models[3,4] and involvement in learning and memory in mouse[5,6] and *Drosophila*[7]. Since neurofibromin is a Ras GTPase-activating protein (GAP)[8-11], a negative regulator of Ras, and elevated Ras activity has been detected in a variety of human tumors, the idea dominates that increased Ras activity resulting from *NF1* mutations plays a crucial role in *NF1* pathogenesis[1,2]. However, it remains to be determined whether Ras regulation is the primary and/or only function of neurofibromin and whether elevated Ras activity can explain all the *NF1* phenotypes. In fact, growing evidence suggests that neurofibromin may be involved in functions other than Ras regulation. First, point mutations identified from patients are not restricted to the GAP-related domain (GRD), which only comprises a small part of *NF1*, and most mutations, including several hotspots, are localized outside of GRD[12]. Second, Ras inhibitors are able to rescue some but not all *NF1*-dependent phenotypes in *NF1*-deficient cell lines[13,14]. Third, neurofibromin can bind with another protein, syndecan[15], in addition to Ras. Fourth, in *Drosophila*, the NF1 protein is involved in regulation of G protein-dependent activation of adenylyl cyclase (AC) and this pathway is important for learning and memory[7], in a neuropeptide response[16] and in regulation of body size[17]. Yet the notion has arisen that the NF1 protein may be functionally different in *Drosophila* and vertebrates even though structurally conserved[2] because previously all reported *NF1* phenotypes have been related to the cAMP pathway in flies. Very recently it has been reported that reduced Ras activity can rescue a circadian rhythm defect in *Drosophila NF1* mutants[18], demonstrating that the *Drosophila* NF1 protein is indeed involved in regulation of Ras activity. For all these reasons, this report investigates whether neurofibromin plays a role in regulation of AC activity in vertebrates. By examining the rescue of a cAMP-dependent fly phenotype by the human *NF1* transgene and direct measurement of AC activity in a mouse *Nf1* knockout, it is shown that

vertebrate neurofibromin is able to regulate a component of G protein-stimulated AC activity in mammals.

We firstly examined whether the human *NF1* gene (*hNF1*) can function in flies. Structurally, the fly NF1 protein is 60% identical to human neurofibromin over its entire sequence of approximately 2800 amino acids[17]. For studying function, we focused on the small body size and adenylyl cyclase activity phenotypes. It has been shown that the body size of flies or the length of pupal cases is smaller or shorter in two independently isolated fly *NF1* mutant alleles, $NF1^{P1}$, a deletion that uncovers the *NF1* locus and a few adjacent genes, and $NF1^{P2}$, a P-element insertion[17]. Moreover, this phenotype can only be rescued by increasing activity of the cAMP pathway, but not by attenuating Ras activity. The ability of *hNF1* to rescue this fly *NF1* phenotype was determined. *NF1* mutants that carry a *UAS-hNF1* transgene (*UAS-hNF1;*$NF1^{P1}$ and *UAS-hNF1;*$NF1^{P2}$) were crossed with two different GAL4 lines (*e22c-Gal4;NF1* and *arm-Gal4;NF1*; see Fly Stocks in the Methods for more details). In the progeny, GAL4 should drive expression of the *UAS-hNF1* transgene in all cells in the mutant background. Measurement of the length of pupal cases indicated that expression of *hNF1* was capable of rescuing the small body size phenotype (Fig. 1a). Both GAL4 lines were similarly effective, suggesting the observed increase in the body size resulted from expression of the *UAS-hNF1* transgene. The defect was almost completely rescued in $NF1^{P2}$ mutant, but only partially rescued in $NF1^{P1}$ even though both mutants appear to be null alleles[17]. Many of our previous studies have been limited to $NF1^{P2}$, because the mutation is caused by an insertion of a P-element in the *NF1* gene that may produce more specific *NF1*-related effects. Unlike $NF1^{P2}$, $NF1^{P1}$ is a deletion that uncovers *NF1* as well as adjacent genes[17] and the phenotype may be more complicated (also see incomplete rescue in the neuropeptide response; see ref.16). This observation indicates that the function of human neurofibromin is conserved in flies, particularly in a function that involves the cAMP pathway.

The above conclusion was further supported by assays of AC activity in flies expressing the *hNF1* transgene. It has previously been shown that the basal level of AC activity remains almost normal, but G-protein stimulated AC activity is reduced in *Drosophila NF1* mutants[7]. This

phenotype can be rescued by acutely induced expression of a *Drosophila NF1* transgene, indicating that the defect is not a developmental consequence of the mutations, but that *NF1* is required for G-protein stimulated AC activity. Here we reveal that expression of the human *NF1* transgene under control of the *Gal4-UAS* system also rescued the AC activity defect in *NF1* mutant flies (Fig. 1b). This result directly shows that the human *NF1* protein is able to interact with AC in the same manner as *Drosophila NF1*.

Next, we directly analyzed whether and how AC activity was affected by neurofibromin in vertebrates. Homozygous knockout mice ($Nf^{-/-}$) were used to determine the effects of loss of neurofibromin function on G protein-stimulated AC activity. Because $Nf1^{-/-}$ mice die at embryonic stage 13.5 (E13.5)[19,20], all biochemical assays were limited to 12-13 day old embryos. At this developmental stage, the brain structures were difficult to separate anatomically. We therefore used the whole frontal lobe to extract the cell membrane fraction in which AC activity was assayed (see Methods). Before examination of mutants, the developmental profile of AC activity in normal mice was determined. We compared the control level of GTPγS- and forskolin-stimulated AC activities in E12.5, newborn (P1), and adult mice. GTPγS constitutively activates the heterotrimeric G_s protein, which in turn stimulates AC activity[21]. Forskolin is a pharmacological agent that can directly stimulate AC activity, therefore bypassing the mechanism of G protein activation[22]. GTPγS and forskolin were both capable of stimulating AC activity at all three developmental stages, but the stimulated levels were much higher in P1 and adult as compared to E12.5 (Fig. 2). In contrast, the basal level of AC activity was more similar in all three developmental stages.

To investigate the effects of *NF1* mutations, analysis was limited to the embryo (E12.5). The basal level of AC activity was similar among wild type, $Nf1^{-/+}$, and $Nf1^{-/-}$ mice (Fig. 3a). However, G protein-activated AC activity (stimulated by GTPγS) was reduced in the homozygous mutant, whereas $Nf1^{-/+}$ and $Nf1^{+/+}$ mice showed a similar response (Fig. 3a). Measurement of cAMP concentrations in these embryos showed that accumulation of cAMP was reduced significantly in $Nf1^{-/-}$ embryos (Fig. 3b), supporting the observation that G-protein

stimulated AC activity is reduced in the mutant. To determine the functional relevance of this GTPγS-stimulated AC activity, we examined the effect of the neuropeptide, pituitary adenylyl cyclase-activating polypeptide (PACAP). It has been demonstrated that this peptide stimulates AC activity via G protein-coupled receptors[23] and PACAP-induced modulation of K^+ currents in *Drosophila* is abolished in fly *NF1* mutants[16]. Here we showed that AC activity was clearly increased in $Nf1^{-/+}$ and $Nf1^{+/+}$ mice to a similar level in response to PACAP, but was not significantly increased in $Nf1^{-/-}$ as compared to their respective basal levels (Fig. 3c). Before we could conclude that neurofibromin regulates AC activity however, there were still a number of issues that needed to be addressed.

First, one could argue that the defect in stimulation results from a reduced level of AC, considering the obvious developmental abnormalities in the homozygous mutant mice[19,20]. To address this concern, we assayed the forskolin response, which stimulates AC activity by bypassing G protein activation. AC activity was strongly stimulated in all genotypes and there was no statistically significant difference in the level of stimulation (Fig. 3d) although it was slightly lower in $Nf1^{-/-}$ mice (see below for further study). Thus, an adequate amount of AC is available to be stimulated in $Nf1^{-/-}$ mice.

The second concern could be that less G proteins are expressed in the homozygous mutant. Since the stimulatory Gα subunit is responsible for direct stimulation of AC activity[24], we evaluated the level of Gα expression by Western blot analysis. There were no visible differences among all three genotypes (Fig. 4).

With all these concerns addressed above, one could still maintain that the defect was a consequence of dying embryos. To examine this possibility, we made embryonic primary neuron cultures. Frontal lobes from different genotypes were dissociated and placed on coated slides (see Methods). One-month old cultures (Fig. 5a) were used to assay AC activity (see Methods). Results were very similar to that observed in *in vivo* assays. The basal level was similar and forskolin significantly stimulated AC activity to a similar level among all three

genotypes. GTPγS-stimulated AC activity showed a reduction in *Nf1*$^{-/-}$ mice, but was still significantly higher than the basal level (Fig. 5b).

Taken together, we have shown that expression of a human *NF1* transgene is capable of rescuing cAMP related phenotypes (small body size and G-protein stimulated AC activity defect) and that neuropeptide- and G protein-stimulated AC activity are reduced in mouse *Nf1*$^{-/-}$ homozygous mutants. These observations lead to the conclusion that neurofibromin is required for G-protein stimulated AC activity in vertebrates. Since GTPγS-stimulated AC activity is rather limited in E12,5 tissues as compared to adults (see Fig. 2), the differences in G protein-stimulated AC activity are relatively small between wild type mouse and *Nf1* mutants (see Fig. 3a). Considering the intrinsic variation between AC assays we were nevertheless able to tighten control of experimental conditions in order to minimize this variation and ensure statistically significant results (see Methods). The conclusion that vertebrate neurofibromin is required for G-protein stimulated AC activity is corroborated by assays of cAMP levels, of PACAP-stimulated AC activity, and of AC activity in cultured neurons. Moreover, the rescue of the fly *NF1* defects by expression of the human *NF1* transgene further strengthens support of this conclusion. Similar to what is observed in *Drosophila* [7], there are also two components in G-protein-dependent activation of AC activity in vertebrates: one is *NF1*-independent and the other is *NF1*-dependent. The results of this study showing *NF1*-dependent regulation of AC activity in vertebrates, and a recent study showing that *Drosophila NF1* regulates Ras activity *in vivo*[18], indicate that *NF1* is not only structurally but also functionally conserved in *Drosophila*, mouse, and humans.

In flies, this *NF1*-regulated AC activity is mainly mediated via the *rutabaga*-encoded AC (Rut-AC)[7], the only one that is sensitive to Ca^{2+}/CaM in *Drosophila*[25] while two types of AC, AC1 and AC8, are sensitive to Ca^{2+}/CaM in vertebrates[26,27]. It remains to be determined whether AC1, which is homologous to Rut-AC, or AC8, or both are involved in mediating *NF1*-regulated AC activity. We should mention that a recent publication has shown that the cAMP concentration is higher in primary Schwann cell cultures isolated from *Nf1*$^{-/-}$ mice in comparison

to wild type cultures[28]. In addition to alteration of AC activity itself, a number of other factors may affect accumulation of cAMP, such as the phosphodiesterase that degrades cAMP, or the secretion of neuropeptides and hormones that stimulate AC activity. Alternatively, neurofibromin may regulate the cAMP pathway differently in Schwann cells and neurons (the present data were generated mainly from central neurons).

It is important to note that heterozygous *Nf1*$^{-/+}$ showed no significant difference in mean AC activity or cAMP concentrations when compared to wild type embryos (see Figs 3 and 5). This implies that the *NF1*-regulated AC pathway may have limited impact on clinical manifestations observed in heterozygous individuals, such as learning deficits[29], while it might contribute to phenotypes that require the loss of heterozygosity[1,2]. It is possible that postembryonic heterozygous *Nf1*$^{-/+}$ mice may show significant differences given the larger G-protein stimulated AC activity observed at later stages of development (see Fig. 2). However, we must point out that the variance in distribution of cAMP concentrations was much larger in heterozygous embryos as compared to wild type and homozygous mutant embryos (see Fig. 3b). Such a distribution appears consistent with the lack of learning deficits observed in a portion of both patients and mice[5,6,29]. Thus, one may not exclude the possible effect on learning of the observed reduction of *NF1*-regulated AC activity.

Methods

Fly Stocks: *NF1*^{P1}, *NF1*^{P2} and K33 flies have a similar genetic background after outcrossing with 2202u (an isogenic line derived from w^{1118})[30] for five generations. Transgenic flies carrying the human *NF1* gene (*hNF1*) were isolated by germline transformation. The *hNF1* cDNA was ligated into the UAS-Gal4 vector pUAST which can be controlled by expression of Gal4 in various tissues. Transformation constructs and pTurbo (encoding the transposase) plasmid DNAs was injected into 2202u embryos according to the method of Rubin and Spradling[31]. All surviving adult flies were individually back-crossed to 2202u and germline transformants were

identified on the basis of a change in eye color. The transgenic UAS-*hNF1* gene is inserted in the second chromosome for the line used in this experiment. The second chromosome GAL4 lines *e22c-Gal4* and *arm-Gal4* express GAL4 ubiquitously in all tissues and at all stages[32,33]. Flies doubly homozygous for *e22c-Gal4;NF1* or *arm-Gal4;NF1* were generated and then crossed to *UAS-hNF1;NF1* double homozygotes for pupal length measurements of progeny and related genetic controls.

Harvesting mouse embryos: All animals described here are on C57BL/6 genetic background (Jackson strain). Embryonic day 12~13 mouse embryos were recovered as described in Brannan *et al.* (see ref. 19) and genotyped by PCR as detailed by Jacks *et al.* (see ref. 20). Frontal brain lobes were dissected in Hank's solution. Brain cells were collected for membrane extraction prior to AC assays or for neuronal culture preparation. Only litters containing all three genotypes, i.e. *Nf1* $^{+/+}$, *Nf1* $^{+/-}$ and *Nf1* $^{-/-}$ were selected for further experimentation.

Neuronal Cell Culture: Frontal lobes were carefully dissected from E12-E13 mice. Embryos were staged according to Theiler[34], and stage-matched embryos were genotyped individually by PCR[20]. Cortical tissues were dissociated to single cell-suspensions by trituration with a fire-polished Pasteur pipette after incubation with 0.25% trypsin/calcium and magnesium-free phosphate-buffered saline (GIBCO) for 5 to 10 minutes at 37 °C. Cortical cells from each embryo were distributed equally on Poly-D-lysine coated 35mm slides. Cells were maintained at 37°C/5% CO_2 and 95% humidity for 30 days until reaching confluence in culture medium consisting of Neurobasal, 0.5mM L-glutamine (Gibco), 50U/ml penicillin (Gibco), 50ug/ml streptomycin (Gibco) and 2% B27 supplement. In B27/Neurobasal, glial growth is reduced to

less than 0.5% of the nearly pure neuronal population[35]. Only confluent neuronal cultures were used for experiments.

Adenylyl Cyclase Assay: The described AC activity assay[36] was modified as follows. Frontal brains from individual embryos (E12.5) or whole fly heads were homogenized using 7ml Tenhroeck tissue grinders (Kontes) for 2 minutes on ice. Membrane fractions were extracted by centrifugation at 178,000g for 10min at 4 °C. Basal AC activities were generated by adding Ca^{++} 0.1 μM and 3 μM Calmodulin (Sigma) to the membrane at 37°C. GTPγs (final concentration 10 μM), PACAP38 (final concentration 1μM), and forskolin (final concentration 100 μM) was incubated with membranes respectively for 20min before assaying stimulated AC activities. For all assays, 10 mM $MgCl_2$ was used. Calcium concentrations were calculated according to MaxChelator v1.31 (see ref. 37). Neuronal cultures were treated with 0.25% Trypsin for 1 minute at 37°C to be collected for homogenization, membrane extraction and AC assay as described above. Statistical analysis was achieved by two-tailed Student's t-test. We want to emphasize that in order to obtain comparable data, it is crucial to keep protein concentrations at a similar level (after extensive exploration, we controlled it in the range of 2 to 5 μg/μl). In particular, we adjusted protein concentrations to be almost identical in all test tubes within the same batch of assays. This is because our data indicated that the protein concentration had profound effect on the calculated rate of AC activity even after the effects of protein concentrations were corrected in the equation. For controlling this and other intrinsic variability, all data from the same batch of assays, including wild type, heterozygous, and homozygous, were included in the analysis.

Cyclic AMP measurement: Frontal brain lobes of embryos were dissected at 4°C and immediately frozen in liquid nitrogen, then kept at –70°C for experimentation. Tissues were homogenized in 500µl of ice-cold Phosphate buffer (Cayman) and 5% trichloroacetic acid (TCA) with Tenhroeck tissue grinders (Kontes). Remove the precipitate by centrifugation at 1,500g for 10min. The cAMP content of the supernatant was measured using a cAMP enzyme immunoassay kit according to the instructions of the manufacturer (Cayman Chemical 581001). The pellets from TCA precipitation were collected for protein determination using Bio-Rad protein assay based on the Bradford dye-binding procedure[38].

G protein density analysis: Mouse brain cells were homogenized as described above and protein concentrations were determined by the method of Bradford et al. (see ref. 38). Forty micrograms of membrane proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (Invitrogen) and then transferred by electrophoresis to nitrocellulose. Immunoblotting was performed with antisera to Gsα (Calbiochem, dilutions 1:1000). Nitrocellulose membranes were incubated overnight with 10% nonfat dry milk in PBS containing 0.1% Tween-20 (0.1% TBS) at 4°C. Blots were washed four times with 0.1% TBS (10 min each) and then incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (Jackson) for 1.5 hr at room temperature. Blots were washed by four 5-min washes with 0.1% TBS, then incubated with Supersignal Chemiluminescent Western blot substrate (Pierce) for 1 min before exposure to X-ray film for 45 seconds.

References

1. Cichowski, K. & Jacks, T. NF1 tumor suppressor gene function: Narrowing the GAP. *Cell* **104**, 593-604 (2001).
2. Zhu, Y. & Parada, L.E. Neurofibromin, a tumor suppressor in the nervous system. *Exp. Cell Res.* **264**, 19-28 (2001).
3. Cichowski, K *et al.* Mouse models of tumor development in neurofibromatosis type 1. *Science* **286**, 2172-2176 (1999)
4. Vogel, K.S. *et al.* Mouse tumor model for neurofibromatosis type 1. *Science* **286**, 2176-2179 (1999).
5. Silva, A.J. *et al.* A mouse model for the learning and memory deficits associated with neurofibromatosis type 1. *Nat. Genet.* **15**, 281-284 (1997).
6. Costa, R. M. *et al.* Learning deficits, but normal development and tumor predisposition, in mice lacking exon 23a of Nf1. *Nat Genet.* **27**, 399-405 (2001).
7. Guo, H. F., Tong, J., Hannan, F., Luo, L. & Zhong, Y. A neurofibromatosis-1-regulated pathway is required for learning in *Drosophila*. *Nature* **403**, 895-898 (2000).
8. Ballester, R. *et al.* The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. *Cell* **63**, 851-859 (1990).
9. Xu, G.F. *et al.* The catalytic domain of the neurofibromatosis type 1 gene product stimulates ras GTPase and complements ira mutants of *S. cerevisiae*. *Cell* **63**, 835-841 (1990).
10. Buchberg, A. M., Cleveland, L. S., Jenkins, N. A. & Copeland, N. G. Sequence homology shared by neurofibromatosis type-1 gene and IRA1 and IRA2 negative regulators of the RAS cyclase AMP pathway. *Nature* **347**, 291-294 (1990).
11. Martin, G. A. *et al.* The GAP-related domain of the NF1 gene product interacts with ras p21. *Cell* **63**, 843-849 (1990).
12. Fahsold, R. *et al.* Minor lesion mutational spectrum of the entire NF1 gene does not explain its high mutability but points to a functional domain upstream of the GAP-related domain. *Am. J. Hum. Genet.* **66**, 790-818 (2000).

13. Kim, H. A., Ling, B. & Ratner, N. Nf1-deficient mouse Schwann cells are angiogenic and invasive and can be induced to hyperproliferate: reversion of some phenotypes by an inhibitor of farnesyl trasfersase. *Mol. Cell. Biol.* **17**, 862-872 (1997).

14. Mahgoub, N. et al. In vitro and in vivo effects of a farnesyltransferase inhibitor on Nf1-deficient hematopoietic cells. *Blood* **94**, 2469-2476 (1999).

15. Hsueh, Y.P., Roberts, A.M., Volta, M., Sheng, M. & Roberts, R.G. Bipartite interaction between neurofibromatosis type I protein (neurofibromin) and syndecan transmembrane heparan sulfate proteoglycans. *J. Neurosci.* **21**, 3764-3770 (2001).

16. Guo, H.F., The, I., Hannan, F., Bernards, A. & Zhong, Y. Requirement of *Drosophila NF1* for activation of adenylyl cyclase by PACAP38-like neuropeptide. *Science* **276**, 795-798 (1997).

17. The, I. et al. Rescue of a *Drosophila NF1* mutant phenotype by protein kinase A. *Science* **276**, 791-794 (1997).

18. Williams, J.A., Su, H.S., Bernards, A., Field, J. & Sehgal, A. A circadian output in *Drosophila* mediated by Neurofibromatosis-1 and Ras/MAPK. *Science* **293**, 2251-2256 (2001).

19. Brannan, C. et al. Targeted disruption of the neurofibromatosis type-1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. *Genes Dev.* **8**, 1019-1029 (1994).

20. Jacks, T. et al. Tumor predisposition in mice heterozygous for a targeted mutation in NF1. *Nat. Genet.* **17**, 353-361 (1994).

21. Pfeufer, T. & Helmreich, E. J. Activation of pigeon erythrocyte membrane adenylate cyclase by guanylnucleotide analogues and separation of a nucleotide binding protein. *J. Biol. Chem.* **250**, 867-876 (1975).

22. Seamon, K. & Daly, J. W. Activation of adenylate cyclase by the diterpene forskolin does not require the guanine nucleotide regulatory protein. *J. Biol. Chem.* **256**, 9799-801 (1981)

23. Spengler, D. et al. Differential signal transduction by five splice variants of the PACAP receptor. *Nature* **365,** 170-175 (1993).

24. Harris, B. A., Robishaw, J. D., Mumby, S. M., & Gilman, A. G. Molecular cloning of complementary DNA for the alpha subunit of the G protein that stimulates adenylate cyclase. *Science* **229**, 1274-1277 (1985).

25. Levin, L.R. et al. The *Drosophila* learning and memory gene rutabaga encodes a Ca2+/Calmodulin-responsive adenylyl cyclase. *Cell* **68**, 479-489 (1992).

26. Wayman, G.A. et al. Synergistic activation of the type I adenylyl cyclase by Ca2+ and Gs-coupled receptors in vivo. *J. Biol. Chem.* **269**, 25400-25405 (1994).

27. Watson, E.L. et al. The type 8 adenylyl cyclase is critical for Ca2+ stimulation of cAMP accumulation in mouse parotid acini. *J. Biol. Chem.* **275**, 14691-14699 (2000).

28. Kim, H. A., Ratner, N., Roberts, T. M. & Stiles, C. D. Schwann cell proliferative responses to cAMP and Nf1 are mediated by cyclin D1. *J. Neurosci.* **21**, 1110-1116 (2001).

29. North, K. et al. Specific learning disability in children with neurofibromatosis type 1: significance of MRI abnormalities. *Neurology* **44**, 878-883 (1994).

30. Yin J. C. et al. Induction of a dominant negative CREB transgene specifically blocks long-term memory in *Drosophila*. *Cell* **79**, 49-58 (1994)

31. Rubin, G. M. & Spradling, A. C. Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348-353 (1982).

32. Duffy, J.B., Harrison, D.A. & Perrimon, N. Identifying loci required for follicular patterning using directed mosaics. *Development* **125**, 2263-2271 (1998)

33. Sanson, B., White, P. & Vincent, J.P. Uncoupling cadherin-based adhesion from wingless signalling in *Drosophila*. *Nature* **383**, 627-630 (1996)

34. Theiler, K. The HouseMouse: Atlas of Embryonic Development. *New York: Springer-Verlag* (1989).

35. Brewer, G. J., Torricelli, J. R., Evege, E. K., & Price, P. J. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J. Neurosci. Res.* **35**, 567-576 (1993).

36. Livingstone, M.S., Sziber, P.P. & Quinn, W.G. Loss of calcium/calmudulin responsiveness in adenylate cyclase of *rutagaba*, a *Drosophila* learning mutants. *Cell* **37**, 205-215 (1984).

37. Bers, D.M., Patton, C.W. & Nuccitelli, R. A practical guide to the preparation of Ca2+ buffers. *Methods Cell Biol.* **40**, 3-29 (1994).

38. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-54 (1976).

Acknowledgement: We would like to thank I. Hakker, J. An, and J. Coblentz for technical help. This work was supported by grants from NIH (NS34779), army (DAMD17-99-1-9500), NF Inc. Mass Bay area and Illinois NF Inc. to YZ.

Figure Legends.

Fig.1. Rescue of body size phenotype and GTPγS-stimulated AC activity by human *NF1* transgene under Gal4-UAS control. **a**, Rescue of body size was assessed by measuring pupal length. Control wild type K33 flies have normal pupal length whereas $NF1^{P1}$ and $NF1^{P2}$ flies have reduced pupal length. The GAL4 lines *e22c-Gal4* and *arm-Gal4*, which express GAL4 in all tissues at all stages[32,33], do not affect pupal length in either the *NF1* mutant or K33 wild type background. The *UAS-hNF1* transgene also does not affect pupal length when *hNF1* is not expressed (not shown). However, when the *UAS-hNF1* transgene was activated by crossing to *e22c-Gal4* or *arm-Gal4* (in $NF1^{P1}$ and $NF1^{P2}$ mutant background),pupal length was significantly ($p< 0.001$; t-test) increased over *NF1* mutant values. (mean±SEM.; n=50 for each genotype). **b**, Rescue of GTPγS-stimulated AC activity was assayed at 10nM Ca^{2+} in membrane

fractions isolated from fly heads. Wild type K33 fly heads show significant stimulation of AC activity by GTPγS whereas *NF1* mutants show no significant stimulation. Activation of the *UAS-hNF1* transgene by either of the GAL4 lines *e22c-Gal4* or *arm-Gal4* (in $NF1^{P1}$ and $NF1^{P2}$ mutant background) resulted in significant ($p<0.01$; t-test) increases, in GTPγS -stimulated AC activity. Each data point is the average of at least three independent experiments ($NF1^{P1}$ n=3; $NF1^{P2}$ n=4; K33 n=7).

Fig. 2. AC activity at different developmental stages. AC activity was assayed using membrane fraction extracted from mouse frontal lobes at each developmental stage, including Embryonic day 12.5 (E12.5), Postnatal day 1 (P1) and Adult (3 month). Extracts from each individual were divided into three. All data were obtained from wild type mice. The number of assays for each data point: n=3. For this as well as all experiments presented below, the concentrations of GTPγS was 10μM and forskolin was 100μM and data are expressed as mean±SEM. Stimulated AC activity is significantly higher in all situations ($p < 0.01$; t-test).

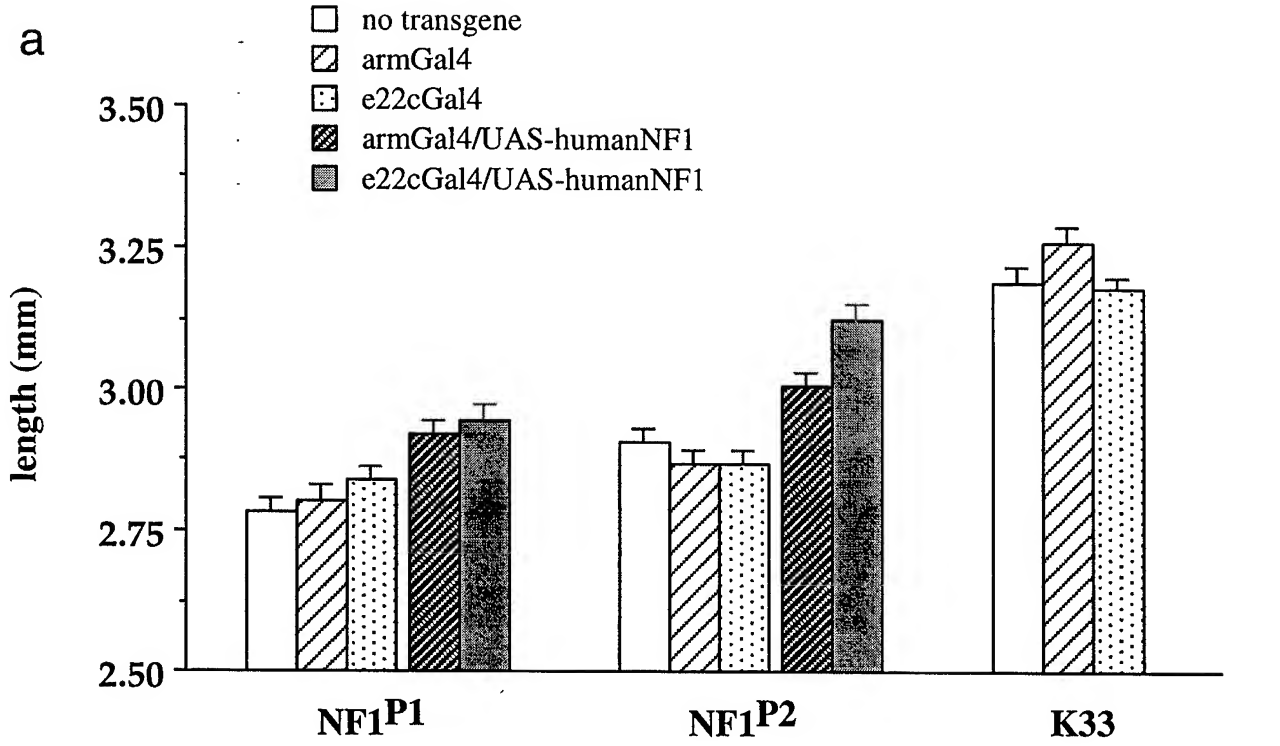
Fig. 3. Reduction in stimulated AC activity in mouse *Nf1* knock out. **a**, GTPγS-stimulated AC activity in wild type, heterozygous, and homozygous mice. AC activity was assayed from extracts of the frontal brain at E12.5. No significant difference (t-test) was found in the basal level, but GTPγS-stimulated AC activity was lower ($p < 0.05$) in homozygous ($Nf1^{-/-}$) utant mice (n=9 embryos) in comparison to both heterozygous ($Nf1^{+/-}$; n=23) and wild type ($Nf1^{+/+}$; n=13). In addition, GTPγS-stimulated AC activity is higher in comparison to the basal level in $Nf1^{-/-}$, but is not statistically significant ($p > 0.3$). To control variability, all AC data were included for analysis only if at least one $Nf1^{-/-}$ embryo was identified in each litter (up to 9 littermates per

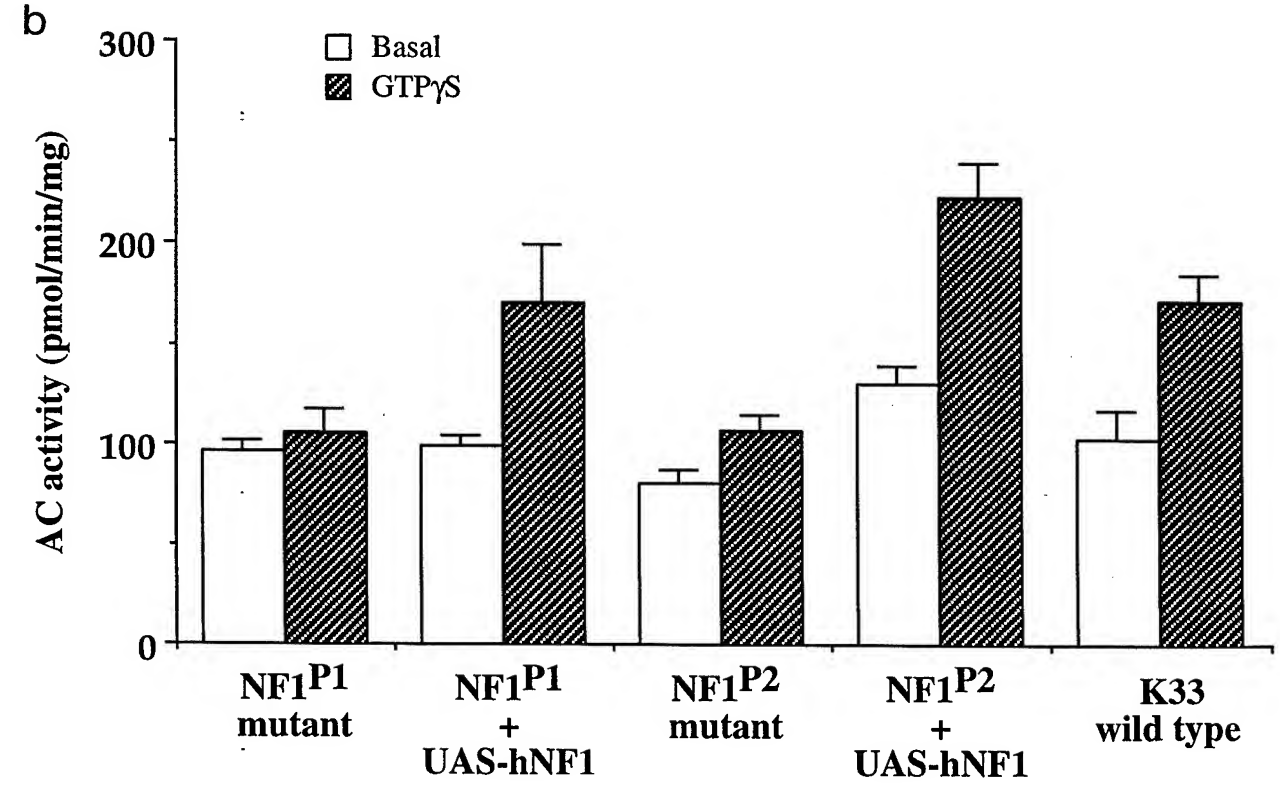
mother). Extracts from littermates were assayed in pairs for both basal level and stimulated AC response. **b**, High variance in *Nf1*$^{+/-}$ and reduced cAMP concentration in *Nf1*$^{-/-}$ frontal brain. cAMP concentration data points from individual embryos (n=5, 10, 4 for *Nf1*$^{+/+}$, *Nf1*$^{+/-}$, and *Nf1*$^{-/-}$ mice respectively) are shown as gray circles. *Nf1*$^{+/-}$ had a higher variance (6.97) than *Nf1*$^{+/+}$ (1.81) and *Nf1*$^{-/-}$ (1.27). Superimposed are mean (cAMP concentration)±SEM.. There was no significant difference between *Nf1*$^{+/+}$ and *Nf1*$^{+/-}$ ($p>0.15$). *Nf1*$^{+/+}$ was significantly higher than *Nf1*$^{-/-}$ ($p<0.001$). *Nf1*$^{+/-}$ was significantly higher than *Nf1*$^{-/-}$ ($p<0.01$). Statistical analysis was achieved by student t-test assuming unequal variance. **c**, PACAP neuropeptide-stimulated AC activity in different genotypes. Assays were performed as above with the addition of 1 μM PACAP. Note the basal level is different from that shown in **a**, but is consistent in each batch of experiments. n=8, 20, and 6 for *Nf1*$^{+/+}$, *Nf1*$^{-/+}$, and *Nf1*$^{-/-}$ respectively. **d**, Normal forskolin-stimulated AC activity in *Nf1* knock out. Again, AC was assayed from the frontal brain of E12.5 embryos. No statistically significant difference was found at the basal level as well as forskolin-stimulated AC activity. n=7, 22, 4 for ***Nf1*** $^{+/+}$, ***Nf1*** $^{+/-}$ and ***Nf1*** $^{-/-}$ mice respectively.

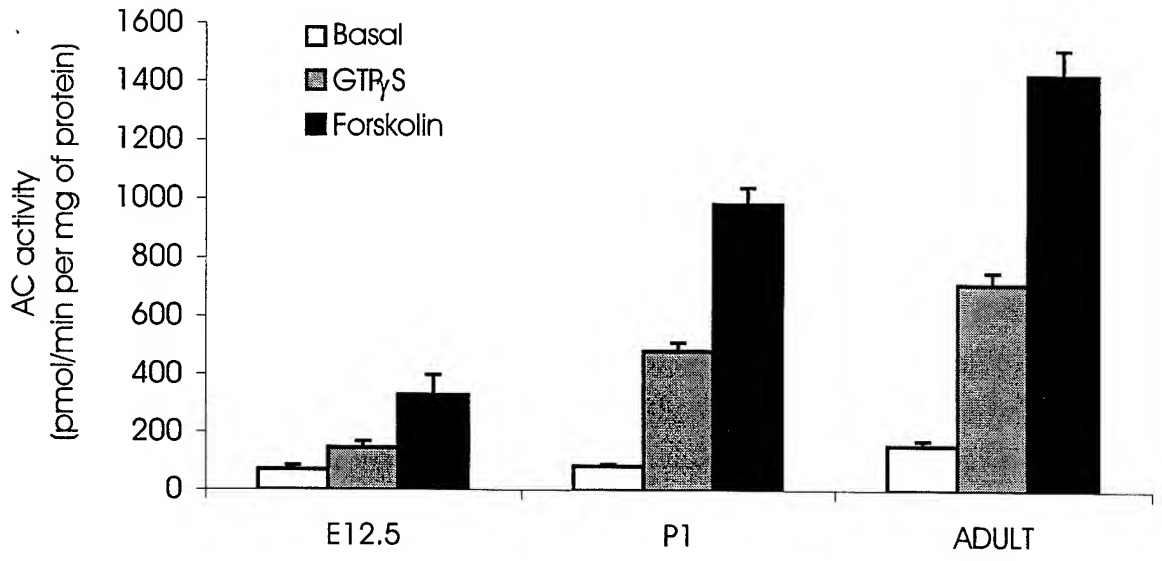
Fig. 4. Effects of *Nf1* knock out on expression of the G protein α subunit. A representative example of immunoblots of Gsα after SDS-PAGE electrophoresis is shown. No obvious disruption was observed. Frontal brain tissues from ***Nf1*** $^{+/+}$, ***Nf1*** $^{+/-}$ and ***Nf1*** $^{-/-}$ littermates were homogenized for Western blot analysis. Molecular weight markers are indicated. Three independent analyses were performed.

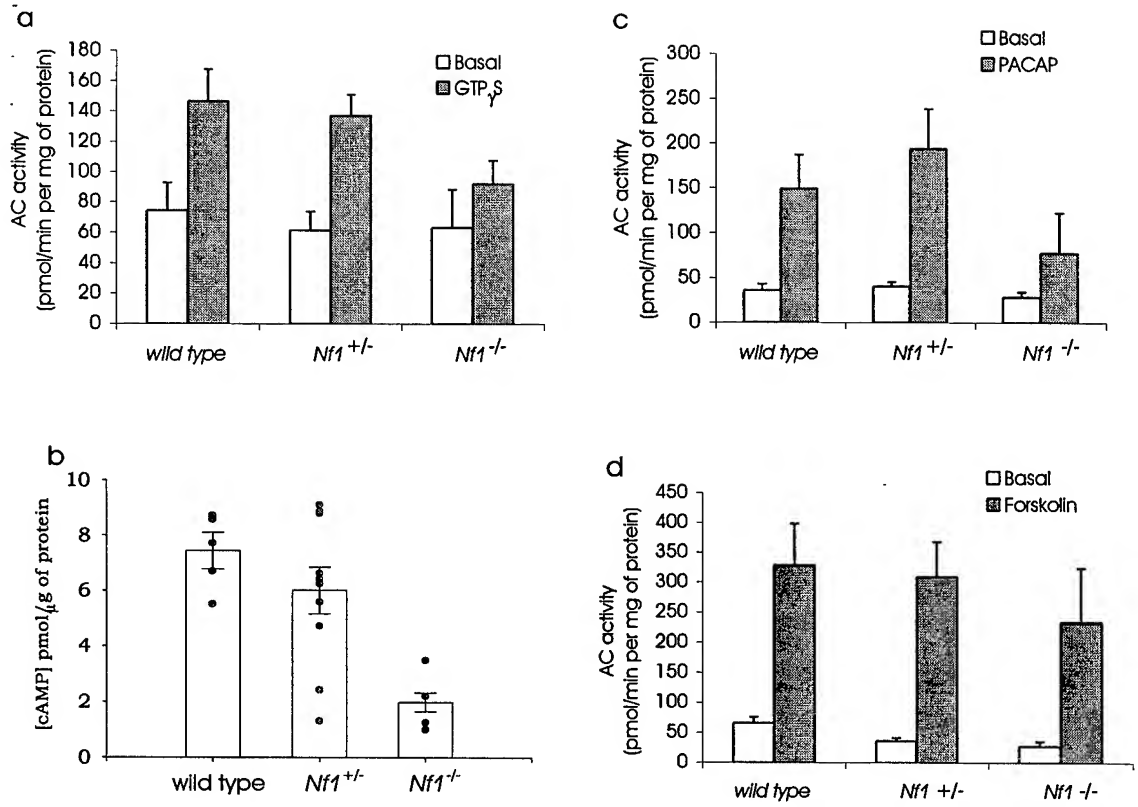
Fig. 5. Altered G protein-stimulated AC activity in primary neuronal cultures disassociated from embryos of mouse *Nf1* knock out.. **a**, Representative examples of cultured neurons derived from

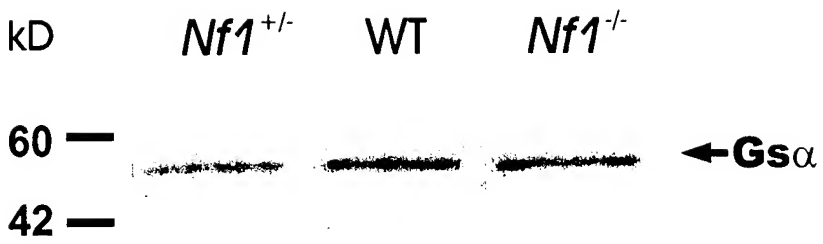
the frontal brain of $Nf1^{+/+}$ and $Nf1^{-/-}$ embryos (E12.5). There was no qualitative difference in growth and morphology observed. **b.** GTPγS- and forskolin-stimulated AC activity in different genotypes. There is no difference observed in the basal and forskolin-stimulated AC activity among all three genotypes. However, GTPγS-stimulated AC activity is significantly lower in $Nf1^{-/-}$ mice in comparison to $Nf1^{+/+}$ ($p < 0.01$) and $Nf1^{-/+}$ ($p < 0.05$). In homozygous mutant, GTPγS-stimulated AC activity is also significantly increased from the basal level ($p < 0.05$). n=7, 11, 11 for *Nf1* $^{+/+}$, *Nf1* $^{+/-}$ and *Nf1* $^{-/-}$ mice respectively.


a
no transgene
armGal4
e22cGal4
armGal4/UAS-humanNF1
e22cGal4/UAS-humanNF1
length (mm)
3.50
3.25
3.00
2.75
2.50
NF1P1
NF1P2
K33

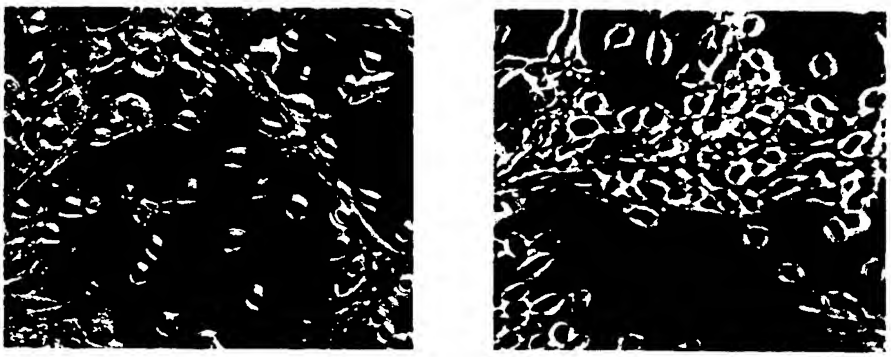

b
Basal
GTPγS
AC activity (pmol/min/mg)
300
200
100
0
NF1P1 mutant
NF1P1 + UAS-hNF1
NF1P2 mutant
NF1P2 + UAS-hNF1
K33 wild type


AC activity
(pmol/min per mg of protein)
1600
1400
1200
1000
800
600
400
200
0
Basal
GTPγS
Forskolin
E12.5
P1
ADULT

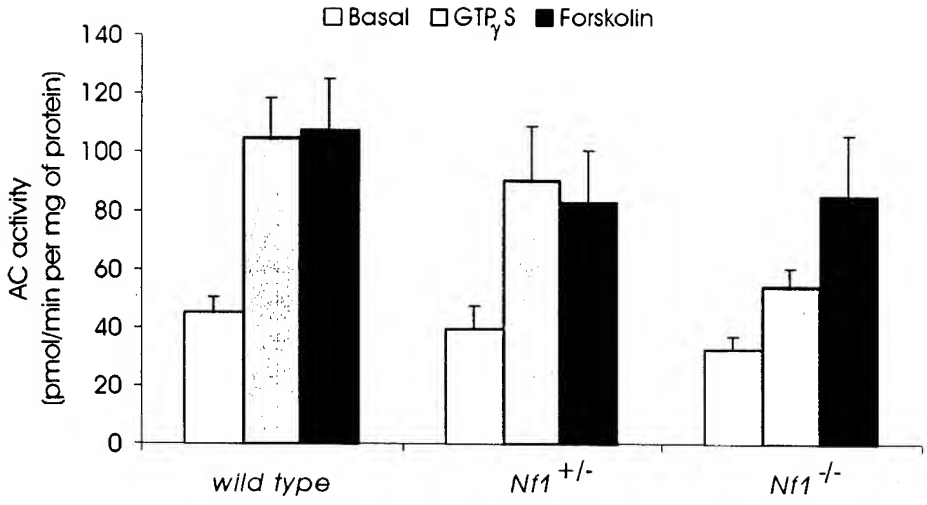

a
AC activity
(pmol/min per mg of protein)
180
160
140
120
100
80
60
40
20
0
Basal
GTPγS
wild type
Nf1 +/-
Nf1 -/-
c
AC activity
(pmol/min per mg of protein)
300
250
200
150
100
50
0
Basal
PACAP
wild type
Nf1 +/-
Nf1 -/-
b
[cAMP] pmol/μg of protein
10
8
6
4
2
0
wild type
Nf1 +/-
Nf1 -/-
d
AC activity
(pmol/min per mg of protein)
450
400
350
300
250
200
150
100
50
0
Basal
Forskolin
wild type
Nf1 +/-
Nf1 -/-


kD
Nf1+/-
WT
Nf1-/-
60
42
←Gsα

a


Nf1 +/-
Nf1 -/-
50uM

b


Basal
GTPγS
Forskolin
AC activity
(pmol/min per mg of protein)
140
120
100
80
60
40
20
0
wild type
Nf1 +/-
Nf1 -/-

Program Number: 780.1 **Day / Time:** Wednesday, Nov. 14, 1:00 PM - 1:15 PM

NEUROFIBROMATOSIS-1 RELATED AGING AND STRESS BEHAVIOR

J.Tong *

Neuroscience Center, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA

Tumor suppressor gene Neurofibromatosis-1 (NF1) encodes a Ras-GTPase activating protein(Ras-GAP). NF1 protein was found to regulate body size and associative learning by integrating Adenylyl Cyclase-cAMP signaling pathways in Drosophila. I found at 23oC and 30oC both NF1 homozygous mutant lines had shortened average life spans by 30% compared with controls. This phenotype was rescued by expressing NF1 protein on the mutant background. Negative geotaxis stressresponse revealed a heat-induced stressrecovery delay in NF1 homozygous mutant flies. Stress recovery delay was correlated with the intensity of the heat stress.Room temperature and cold stress experiments showed little difference between NF1 mutants and controls. The phenotype could be partially rescued by expressing NF1 gene prior to heat stress.Mutants involved in AC-cAMP pathways and Ras pathways were tested to dissect the signaling alteration causing these behavioral changes.
Supported by: Ellison Medical Fund and US Army Grant